

INACTIVATION OF NF- κ B INHIBITOR I κ B α : UBIQUITIN-DEPENDENT PROTEOLYSIS AND ITS DEGRADATION PRODUCT

Exhibit C

Chou-Chi H. Li¹, Ren-Ming Dai and Dan L. Longo¹

Biological Carcinogenesis and Development Program, SAIC-Frederick,
¹Biological Response Modifiers Program, Division of Cancer Treatment,
National Cancer Institute-Frederick Cancer Research and Development Center,
Frederick, MD 21702-1201

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SUMMARY : In most cells, the inactive dimeric NF- κ B complexes are retained in the cytoplasm by binding to a group of inhibitory proteins, I κ B. In response to extracellular stimuli, I κ B is rapidly phosphorylated and degraded, thus, liberating the active NF- κ B. To investigate the mechanisms involved, we have developed a cell-free system to study the degradation of the prototype I κ B protein, I κ B α . In this *in vitro* assay, ubiquitin, proteasome-containing S100 fraction and ATP are required for the proteolysis of I κ B α . Both bound and free forms of I κ B α isolated from intact cells can be degraded through this pathway. We also identified polyubiquitinated I κ B α molecules and N-terminal truncated I κ B α degradation product(s) both *in vivo* and *in vitro*. We conclude that the inactivation of I κ B α occurs through a series of processes including phosphorylation, ATP-dependent ubiquitin conjugation and proteasome-mediated proteolysis. © 1995 Academic Press, Inc.

INTRODUCTION : Nuclear factor κ B plays a central role in the regulation of immune, inflammatory, and adhesion responses, and activation of several viruses, including human immunodeficiency virus (reviewed in ref. 1-6). The active NF- κ B factor is a homo- or hetero-dimer consisting of members of the Rel/NF- κ B family proteins. The family includes NFKB1(p105/p50), NFKB2 (p100/p52), RelA (p65), c-Rel, RelB, and Drosophila dorsal and diph proteins. These proteins share structural and functional similarities in their N-terminal 300 amino acids, termed Rel homology domains, which are essential for DNA-binding, nuclear localization and dimerization functions. Unlike many other transcription factors that are localized in the nucleus, the NF- κ B dimeric factor is sequestered in the cytoplasm of most cells through binding to a group of inhibitor proteins (I κ B), including the prototype I κ B α . I κ B proteins share homologies in their C-terminal domains which contain several ankyrin repeats, thought to be involved in protein-protein interactions. When cells are activated by a variety of stimuli, e.g. virus, bacteria, radiation, oxidants and stress, I κ B α is phosphorylated and quickly degraded (7-14). The dimer is released and moves to the nucleus, where it binds to the decameric κ B binding site, and regulates the transcription of

* corresponding author. Fax:(301) 846-6107.

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the target gene. Recently, Palombella et al. (9) showed that the processing from the inactive p105/p65, in which p105 is the precursor of p50 and behaves as an inhibitor to the active p50/p65 dimer, occurs through a ubiquitin-proteasome (Ub-Pr) pathway. For the prototypical complex p50/p65/I κ B α , although it has been shown that I κ B α degradation is sensitive to proteasome inhibitors (9, 10), the molecular mechanism has not been well elucidated.

MATERIALS AND METHODS

Immunoprecipitation and Western Blot (Immunoblot) Analyses : Human Jurkat T cells and CA46 B cells (15) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 units/ml penicillin and 50 μ g/ml streptomycin. Jurkat cells were stimulated with PMA (100 ng/ml) and PHA (5 μ g/ml) for 20 min, lysed and subjected to Western blot analyses (16-18). CA46 cells were metabolically labeled with [35 S]-methionine/cysteine for 3 h and lysed, the lysates were immunoprecipitated (16-18). Two I κ B α antisera (A and B) (17) were raised against independently synthesized C-terminal peptides (residues 300-317) (19). Serum B was used in Fig. 1A, and the antiserum was affinity-purified using the Proton kit (Multiple Peptide System). Ub antiserum was purchased from Sigma. All lysis buffers contained protease inhibitor cocktail including 1% aprotinin, 40 μ g/ml Tos-Phe-CH₂Cl (TPCK), 5 μ g/ml Tos-Lys-CH₂Cl (TLCK), 70 μ g/ml phenylmethylsulfonyl fluoride (PMSF), 5 μ g/ml Leupeptin and 0.01 u/ml Ca⁺⁺-induced protease inhibitors (Sigma).

In vitro degradation assay : The substrate I κ B α was *in vitro* transcribed and translated in a reticulocyte lysate system (Promega) with [35 S]-cysteine from a Bluescript-I κ B α expression plasmid (20). Equal amounts (2-5 μ l) of translated I κ B α were incubated at 37°C, with 30-50 μ g of S100 fraction (9, 21) extracted from CA46 cells as the enzyme source, and 6 μ g of dialyzed yeast or bovine Ub (Sigma). Each reaction was carried out in a total volume of 50 μ l containing 12 mM Tris-HCl, pH 7.5, 60 mM KCl, 3.5 mM MgCl₂, 5 mM CaCl₂, 1 mM DTT, and 1 mM ATP when desired. Master reaction mixture without S100 was prepared and aliquoted into different tubes on ice. At different time points, S100 was added to individual tubes to start the reaction at 37°C. All the reactions were simultaneously terminated by boiling the samples in the gel loading buffer. The reactions were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western transfer and autoradiography. Untreated rabbit reticulocyte lysate (Promega) and S100 extracted from HeLa or a number of human B cell lines were also used for enzyme sources and all gave similar results. [125 I] conjugation of Ub was performed as described (22). Proteasome depleted (Pr⁻) or enriched (Pr⁺) fraction was extracted as published (9, 23). All inhibitors were purchased from Sigma or Boehringer Mannheim Corp.

GST-I κ B α fusion protein : Glutathione-S-transferase (GST)-I κ B α expression plasmid was constructed by inserting the EcoR1 fragment of I κ B α (20) into pGEX-4T-2 vector (Pharmacia LKB Biotechnology Inc.). The GST-I κ B α fusion protein was prepared according to the manufacturer.

RESULTS : We have previously shown that when the total cell lysate extracted from unstimulated Jurkat cells was immunoblotted with I κ B α antiserum, several high-molecular-mass proteins were detected at low levels (17). To further characterize these proteins, Western (immuno-) blot analysis was performed on lysates extracted from PMA/PHA-stimulated Jurkat cells. As shown in Fig. 1A, in addition to the 36 kD-I κ B α , multiple bands in a ladder-like pattern were detected at an elevated level (left lane). The majority of these proteins were not detected when the analysis was carried out in the presence of competing

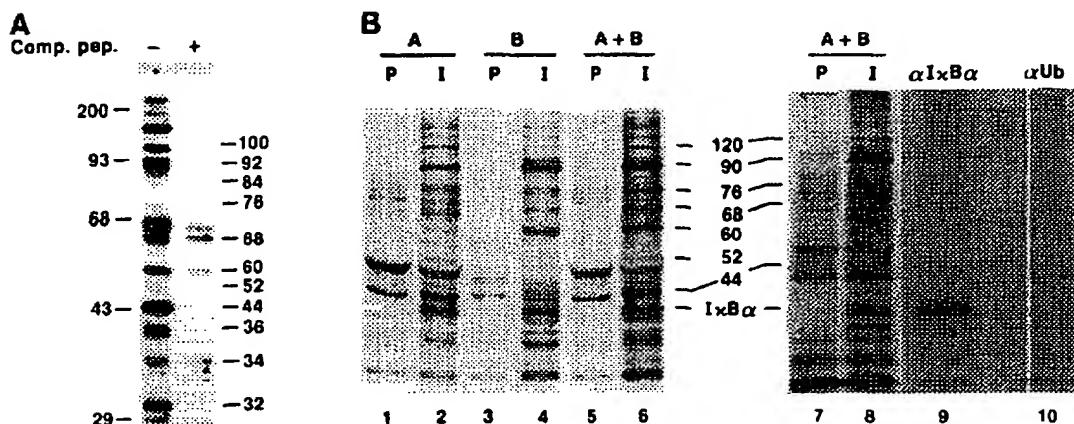


Fig. 1. Detection of ubiquitinated I κ B α in cells.

(A) PMA/PHA-stimulated Jurkat cells were lysed and subjected to Western blot analysis using affinity purified anti-I κ B α immune serum B in the absence (left lane) or presence (right lane) of the competing peptide. Molecular size markers are on the left and the apparent sizes for individual proteins are marked on the right. (B) CA46 cells were metabolically labeled with [35 S]methionine/cysteine and lysed. The lysates were immunoprecipitated with anti-I κ B α immune serum A (lane 2), B (lane 4), mixture of A and B (lanes 6, 8), or the corresponding preimmune sera (lanes 1, 3, 5, 7). For the reprecipitation, washed immune complex (lane 8) was boiled and reprecipitated (25) with anti-I κ B α (lane 9) or anti-Ub (lane 10) serum.

peptide, i.e. the antigenic peptide used to generate the antiserum (right lane). Interestingly, the size increment between each of these multiple bands was about 8 kD, the size of the Ub molecule (reviewed in ref. 24), suggesting that these larger species could be polyubiquitinated I κ B α s. Radio-immunoprecipitation performed on [35 S]-metabolically labeled CA46 cells, which express constitutively active NF- κ B, also showed a similar result (Fig. 1B, lanes 1-8). Using two anti-I κ B sera (A and B), multiple bands were observed, again separated by approximately 8 kD. Moreover, when the I κ B α immune complex (lane 8) was boiled and reprecipitated (24) with either I κ B α (lane 9) or Ub antiserum (lane 10), a number of bands were recognized by both antisera, strongly suggesting that they represent polyubiquitinated I κ B α s. Interestingly, 34 and 32 kD proteins were also specifically detected in both immunoblot (Fig. 1A) and immunoprecipitation (Fig. 1B, lanes 4, 6). Since the cells were lysed in the presence of protease inhibitor cocktails that inhibit the Ub-Pr pathway (also see Fig. 2C), these small I κ B α s are probably degradation products present in the intact cells (also discussed in Fig. 4C).

To elucidate the mechanisms involved in I κ B α degradation, we developed an *in vitro* I κ B α degradation assay, in which I κ B α , as the substrate, and S100, as the enzyme source, were incubated with purified Ub at 37°C (Fig. 2). As [35 S]-cysteine labeled, *in vitro*-translated I κ B α (lane 1) was used, most of the I κ B α was degraded within a few minutes (Fig.

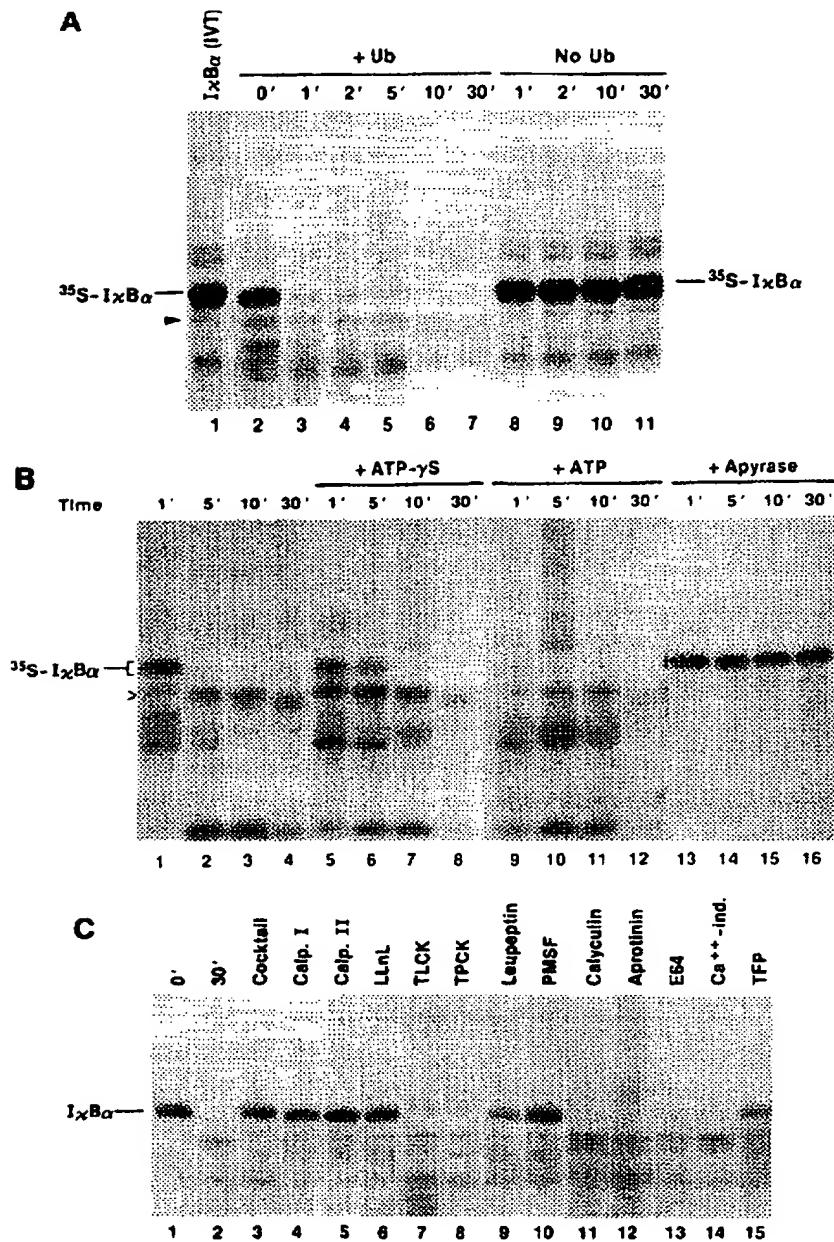


Fig. 2. *In vitro* assay of IκBα degradation.

(A) Ub dependence of IκBα degradation. [³⁵S]cysteine-labeled IκBα (lane 1) was assayed for degradation with (lanes 2-7) or without Ub (lanes 8-11) for various periods of time. The filled arrowhead marks the 34 kD IκBα. (B) ATP dependence of IκBα degradation. Reactions were carried out with Ub in the presence of ATP-γS (2 mM), or ATP (1 mM), or apyrase (10 u/reaction) for indicated periods of time. Arrowhead marks the 34 kD degradation product. (C) Inhibitor study of IκBα degradation. ³⁵S-labeled IκBα was used in 30 min degradation assay in the absence (lanes 1, 2) or presence of various inhibitors (lanes 3-15). All reactions were heated, and analyzed by SDS-PAGE followed by Western transfer and autoradiography.

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2A, lanes 2-7). When the same reactions were performed without Ub, the level of I κ B α was essentially unchanged (lanes 8-11), demonstrating that Ub is a critical requirement for I κ B α degradation. No degradation was detected when the assay was performed without S100 (data not shown), indicating that I κ B α degradation was not due to nonspecific protease activities present in the Ub preparation.

Since the Ub-Proteasome pathway requires ATP for energy, we further carried out the assay with the addition of ATP- γ S, ATP, or Apyrase (Fig. 2B). It is clear that addition of ATP accelerated the degradation of I κ B α (compare lanes 9-12 with 1-4), whereas addition of ATP- γ S, an unhydrolyzable ATP analog, decreased the rate of degradation (compare lanes 5-8 with 1-4). Apyrase, which destroys ATP, completely blocked the degradation (lanes 13-16). These data clearly demonstrate that an ATP-dependent Ub-Pr pathway is involved in I κ B α degradation.

In order to further identify the components of this process, degradation assays were carried out in the presence of various inhibitors (partially shown in Fig. 2C). The reagents that blocked the I κ B α degradation include the proteasome inhibitors peptide aldehyde Ac-LLnL-CHO (100 μ M) (Sigma), calpain inhibitors I (100 μ M) and II (400 μ M) (Boehringer Mannheim Corp.), and serine/cysteine protease inhibitors, TPCK (100 μ M), TLCK (300 μ M), leupeptin (100 μ M), and PMSF (200 μ M). Although TPCK and TLCK partially inhibit the reaction and are not considered proteasome-specific inhibitors, they probably interfere with the phosphorylation required for the proteolysis (11-13). Interestingly, trifluoperazine (TFP) (26), a calmodulin antagonist, inhibited I κ B α degradation (lane 15).

In addition to the free *in vitro* translated I κ B α (as shown in Fig. 2), I κ B α associated with NF- κ B is also degraded through the *in vitro* Ub-Pr pathway (Fig. 3A). B cells were metabolically labeled with [35 S]-methionine/cysteine, and the cell lysates were immunoprecipitated with anti-NF- κ B to isolate the associated I κ B α , or with anti-I κ B α to isolate the free and the bound I κ B α molecules. When both bound I κ B α (lane 3) and total I κ B α (lane 5) isolated from cells were subjected to *in vitro* degradation assays (lanes 6-9), both were degraded within minutes (lanes 7, 9). As has been previously reported (9), we also observed that p105 was rapidly processed (compare lanes 3 and 7). A similar assay carried out with a reduced amount of S100 (Fig. 3B), which attenuated the Ub-Pr pathway, showed the appearance of I κ B α with slightly less gel mobility (lane 3). The slower gel mobility is probably a result of differential phosphorylation (17), because both forms of I κ B α were reactive to the I κ B α antiserum (data not shown). This result suggests that phosphorylation of I κ B α precedes the Ub-Pr pathway. Interestingly, a 90-kD cellular protein has been reproducibly detected in I κ B α immune complexes (ref. 17 and Figs. 1B, 3A, 3B). This protein appears to be relatively resistant to the Ub-Pr proteolysis (Fig. 3A, lane 9). We are currently identifying this protein.

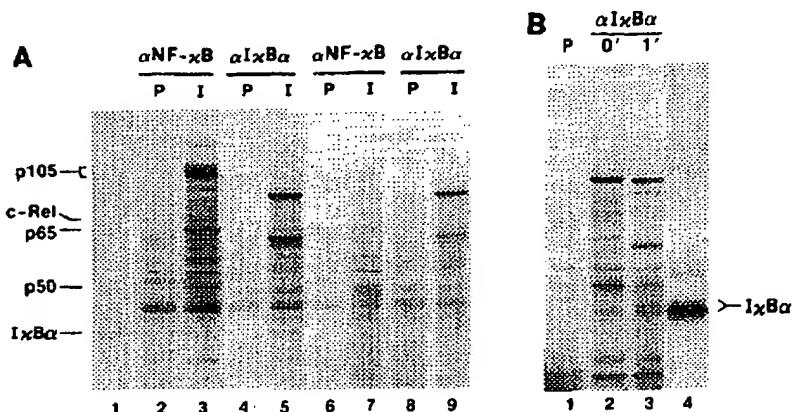


Fig. 3. Degradation of both free and bound forms of I κ B α isolated from cells.
 (A) [35 S]methionine/cysteine labeled CA46 cell lysates were immunoprecipitated with anti-NF- κ B preimmune (lane 2) and immune (lane 3) sera, or anti-I κ B α preimmune (lane 4) and immune (lane 5) sera. Half of each immune complex was subjected to the degradation assay for 5 min (lanes 6-9). [35 S]-labeled *in vitro* translated I κ B α is shown in lane 1. NF- κ B antiserum is a mixture of antisera against p105N, p65, and c-Rel (16-18). I κ B α antiserum is the combined sera A and B described in Fig. 1(B). (B) [35 S]methionine/cysteine labeled CA46 cell lysates were immunoprecipitated with anti-I κ B α preimmune (lane 1) and immune (lanes 2, 3) sera. The washed complexes were subjected to an attenuated degradation assay for 1, 0, and 1 min in lane 1, 2, and 3, respectively. The attenuated assay was carried out using 40% of the amount of S100 as in the normal assay. The *in vitro* translated [35 S]-labeled I κ B α is shown in lane 4.

The larger forms of I κ B α observed as smears in Fig. 2B, lanes 7 and 10 in the *in vitro* assays probably represent ubiquitinated I κ B α molecules. To identify these proteins, we incubated [35 S]-labeled I κ B α and [125 I]-labeled Ub with proteasome depleted S100 (Pr $^+$) in the presence of ATP- γ S (Fig. 4A, lanes 1-3). Both Pr $^+$ and ATP- γ S were used to slow the ubiquitination process and block the proteolysis. Increasing amounts of [125 I] were incorporated in a ladder-like pattern into proteins larger than I κ B α , representing the ubiquitinated I κ B α molecules (Fig. 4A, lower panel, lanes 1-3). This [125 I] incorporation required Ub (compare lanes 1-3 with 4-6) and did not result from ubiquitination of the background proteins present in the Pr $^+$ fraction, because the same assay performed without I κ B α yielded no [125 I] incorporation (lanes 7-9). The ubiquitination of I κ B α was further demonstrated in a Ub-Pr $^+$ reaction performed on the GST-I κ B α fusion protein (Fig. 4B). The high-molecular-mass, polyubiquitinated I κ B α proteins were detected in a time-dependent manner.

Although it has been well established that activation of NF- κ B requires I κ B α degradation, no degradation products have been identified. The detection of 34 kD and 32 kD I κ B α molecules in intact cells (Fig. 1) suggests that they may be products or intermediates produced in the degradation process. In the *in vitro* assays, a smaller form (34 kD) of I κ B α accumulated during the reaction (see Fig. 2, arrow heads). To further

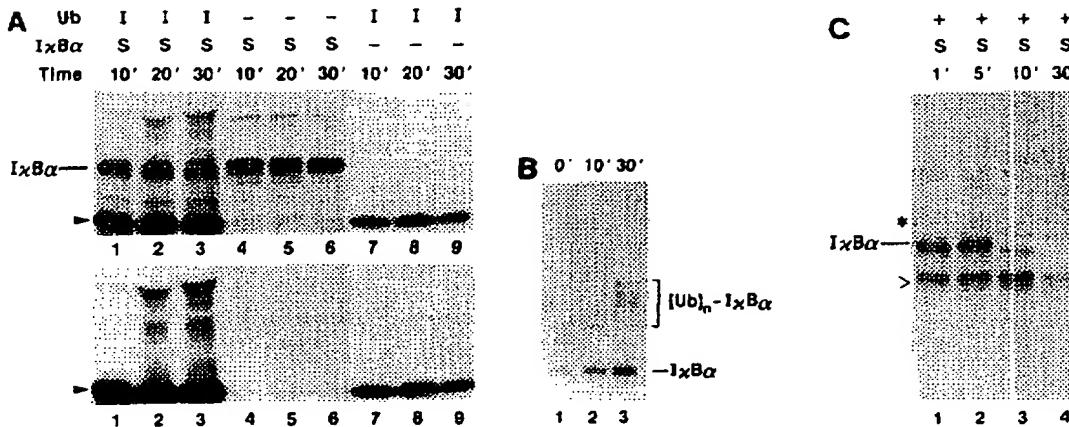


Fig. 4. Identification of ubiquitinated IκBα and degradation product *in vitro*.
(A) *In vitro* assays were carried out as indicated on the top of the panel (+: unlabeled; -: absence; I: ^{125}I ; S: ^{35}S). Reactions were carried out and analyzed as described in Fig. 2 except that Pr⁻ fraction, instead of S100, was used as the enzyme source. Two X-ray films were used in autoradiography. The film right on top of the filter showed both ^{125}I and ^{35}S activities (top panel), and the second film showed only the ^{125}I activity (bottom panel). ^{125}I activity specific to unincorporated Ub is marked as a filled triangle. **(B)** Glutathione-Sepharose beads containing GST-IκBα fusion protein was subjected to Ub-Pr⁻ reaction for 0 (lane 1), 10 (lane 2) or 30 (lane 3) min. The washed beads were analyzed by SDS-PAGE. Western transfer and immunoblotted with anti-IκBα immune serum. **(C)** Reactions were carried out as described in (A) and immunoprecipitated with anti-IκBα (serum A+B) in the presence of protease inhibitor cocktail. The star and the arrowhead mark the 44 kD ubiquitinated IκBα (lane 2) and the 34 kD degradation product, respectively.

characterize these proteins, *in vitro* reactions were immunoprecipitated with anti-IκBα (Fig. 4C). While a larger form of IκBα, ubiquitinated IκBα, was detected in 5 min (lane 2), 34 kD IκBα appeared to accumulate then degrade during the assay (lanes 3, 4). This 34 kD IκBα was detected by the antiserum raised against the C-terminus of IκBα, both *in vivo* (Fig. 1) and *in vitro* (Fig. 4C), but not by the antiserum recognizing the N-terminus of IκBα (data not shown). This result suggests that the 34 kD-IκBα lacks the N-terminal portion which is probably the initial target for degradation.

DISCUSSION

We have previously (17) shown that NF-κB/Rel family members and IκBα, which is constitutively phosphorylated, are physically associated phosphoproteins containing phosphorylated serine and threonine residues. Recently, we (18) and others demonstrated that, in addition to IκBα, phosphorylation of NF-κB family proteins, such as p50 (18), p65 (27), c-Rel (28) and dorsal (29), also plays a critical role in NF-κB activation. Moreover, after stimulation most of the hyperphosphorylated IκBα still remains in the complex, suggesting that phosphorylation of IκBα is not sufficient to cause dissociation of the inhibitor from the complex (7-14, 17). In our *in vitro* assays, a slightly slower migrating form of

$I\kappa B\alpha$, presumably the hyperphosphorylated $I\kappa B\alpha$ (17), reproducibly appeared shortly after the Ub-Pr pathway started (Fig. 2B, lane 6) and before the ubiquitination took place (lane 7). *In vitro* assays carried out with a reduced amount of S100, which attenuated the Ub-Pr pathway, also showed rapid phosphorylation of 36 kD- $I\kappa B\alpha$ (Fig. 3B). These results suggest that phosphorylation precedes the process of ubiquitination and proteolysis. Our previous detection (17) of highly phosphorylated, larger-sized $I\kappa B\alpha$ s in a ladder-like pattern, indicative of ubiquitinated $I\kappa B\alpha$ s, in intact cells supports this model. While this manuscript was being prepared Chen et al. (30) showed that signal-induced phosphorylation of $I\kappa B\alpha$ precedes ubiquitination and targets it to the Ub-Pr pathway, confirming the model.

In this report, we have demonstrated the Ub requirement for the degradation of $I\kappa B\alpha$ and identified the ubiquitinated $I\kappa B\alpha$ s and the degradation product both *in vivo* and *in vitro*. The lack of ready detection of these ubiquitinated $I\kappa B\alpha$ proteins by other researchers may be due to the following: 1. Antiserum directed against the N-terminal $I\kappa B\alpha$ was used in most experiments. The N-terminus of $I\kappa B\alpha$ has been shown to be phosphorylated (20) upon activation, and has 7 of 8 lysine residues of the $I\kappa B\alpha$ molecule (19), presumably the ubiquitination sites. It is probably the major region that is highly modified in response to stimulating signals, and serves as the target for later proteolysis (20, 30, 31 and this study). Therefore, the antiserum raised against the N-terminal synthetic peptide of $I\kappa B\alpha$ may not have ready access to this highly modified N-terminus of $I\kappa B\alpha$. 2. The Ub-Pr pathway takes place so fast that the modified $I\kappa B\alpha$ s are degraded in the analysis process unless appropriate protease inhibitors are included. 3. It is more difficult to detect ubiquitinated $I\kappa B\alpha$ s in cells that are induced to activate NF- κB than in cells that have constitutively active NF- κB , e.g. B cell lines (data not shown). 4. Because of the ubiquitous nature and evolutionary conservation of Ub, it is extremely difficult to raise a good Ub antiserum. Although obtaining the ultimate and unambiguous proof for the ladder-patterned $I\kappa B\alpha$ s as the multiple Ub conjugates has been hampered by the lack of such an antiserum (Fig. 1), a similar ladder-like pattern was detected in another report (7). The first three reasons could also partially explain why the N-terminal truncated $I\kappa B\alpha$ degradation product was not readily detected. However, a recent report (32) showing the presence of N-terminal truncated p40, the avian homolog of $I\kappa B\alpha$, in v-rel transformed cells, supports our finding. Our data suggest that the 34 kD (and probably also the 32 kD) $I\kappa B\alpha$ (s) detected *in vivo* and *in vitro* is (are) $I\kappa B\alpha$ degradation product(s) lacking the N-termini. It has been suggested that a chymotrypsin-like protease is probably involved in the proteolysis of $I\kappa B\alpha$ (8, 10-14, 33). Consistently, cleavages at chymotrypsin sites, residues 20 and 66 of $I\kappa B\alpha$ (33), would produce proteins similar in size to those we have detected. Since the truncated $I\kappa B\alpha$ missing the N-terminal region, which contains the signal responsive sequence, is still capable of inhibiting the DNA-binding activity of p65 (33, 34), it would behave as a dominant negative mutant. These degradation products may further play a role in the negative regulation of NF- κB after the maximal DNA-binding activity is reached.

Taken together, we propose the following model. In response to stimulation, the entire NF- κ B complex, e.g. p50/p65/I κ B α becomes hyperphosphorylated (18, 27-29). The induced phosphorylation of I κ B α does not lead to its immediate dissociation from the complex; rather, it signals for a rapid N-terminal polyubiquitination and subsequent degradation. Both the free and the NF- κ B-bound forms of I κ B α can be degraded through the proteasome-mediated proteolysis *in vitro*. At least one of the degradation products is a 34 kD protein that lacks the N-terminal domain. The degradation of I κ B α liberates the NF- κ B dimer that translocates to the nucleus, where the dimer binds to the cognate κ B site and regulates the transcription of the target gene. It is of interest that trifluoperazine (TFP), a calmodulin antagonist (26), inhibited I κ B α degradation (Fig. 2C, lane 15), suggesting that a Ca⁺⁺/calmodulin dependent enzyme reaction may be critical for the Ub-Pr pathway involved in I κ B α degradation. We are currently investigating this aspect. In conclusion, it appears that phosphorylation, ubiquitination and proteolytic degradation of I κ B α are both necessary and sufficient for inactivation of I κ B α . Whether they are sufficient for activation of NF- κ B remains to be determined because phosphorylation of NF- κ B dimers is also essential (18, 27-29).

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